

## Supplementary Material for

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**“The role of HSP70 on ENPP1 expression and insulin receptor activation”**

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### SUPPLEMENTARY METHODS

#### *RNA probes*

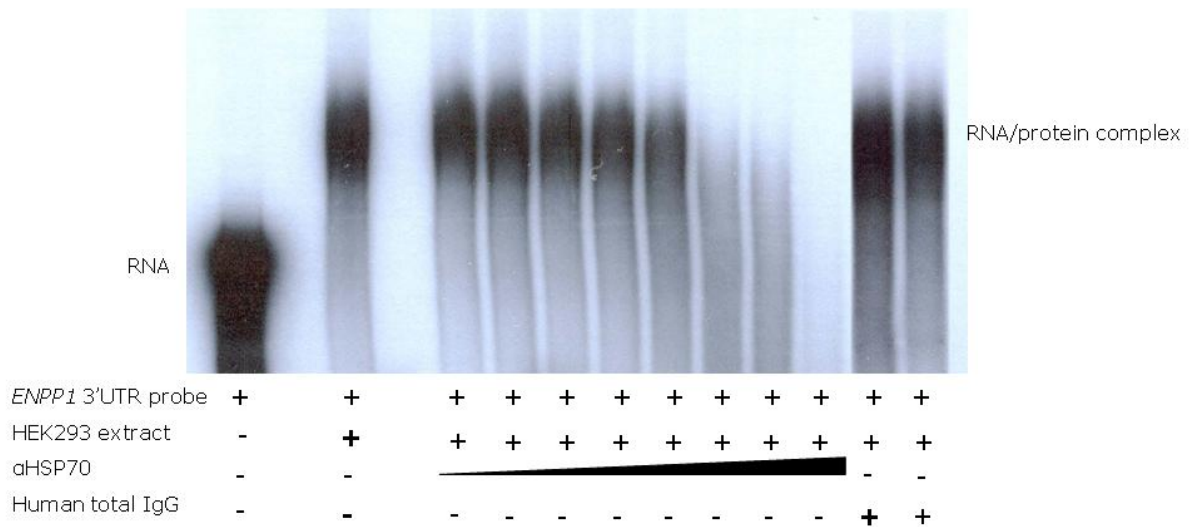
Nucleotides (nt) 2750 to nt 3176 of the *ENPP1* cDNA was amplified from plasmid prk7-*ENPP1* (kindly provided by Dr. Ira Goldfine, San Francisco, CA), cloned into pCRII-TOPO vector (Invitrogen) and then excised with EcoRI, and subcloned into pGEM-3Zf(+) vector (Promega) downstream of a T7 RNA polymerase promoter. M13 forward and M13 reverse primers were used to obtain a fragment including the *ENPP1* and the PCR product used for preparation of  $^{32}\text{P}$ -labeled RNA probe by using Maxiscript T7 Kit (Ambion). The RNA probe was purified by 8M urea polyacrilamide gel electrophoresis (PAGE).

#### *Cell culture and cell extract*

Human embryo kidney 293 (HEK293) cells and human hepatocarcinoma HepG2 cells were grown in DMEM/F12 medium containing 10% FBS at 37°C with 5% CO<sub>2</sub>. Cells were grown for 48 hours in 75-cm<sup>2</sup> culture flasks, seeded into six well/plates and cell extracts obtained as previously described by Mondino A, Jenkins MK (1995) *J Biol Chem* 270: 26593-26601.

**Supplementary figure 1. Sequence comparison of mouse *Enpp1* and human *ENPP1* 3'UTR.** Nucleotide sequences of human *ENPP1* (M57736) and mouse *Enpp1* (J02700) from position 2750 to 3493 are shown. Nucleotides identical for both mouse and human *ENPP1* are indicated by asterisk. Primers used to generate DNA template for transcribing RNA probe are underlined.

mouse	CTACCAGGACCGACAAGAGTCAGTTTCAGAACTGCTGAGGTTGAAAAACACATTTGCCAAT	2810
human	CTATCAACAAAGAAAAGAGCCAGTTTCAGACATTTTAAAGTTGAAAACACATTTGCCAAC	2775
	*** * * * * *	
mouse	CTTCAGC CAA GAAGAC TGA TTGTT TTTTAT TAAAAA CAAAAG AAAA CAAACAC	2870
human	CTTTAGCC CAGAAGAC TGA TTGTT TTTTAT CCC-----CAACAC	2816
	*** **** * *	
mouse	CATAGATCCTTTTGAAAGAGCTTTATATTTGATACAGTCCTCTA---CACTTTTGCAATT	2926
human	CATGAATCTTTTGAGAGAACTTATATTTTATATAGTCCTCTAGCTACACTATTGCATT	2876
	*** **** * *	
mouse	GTTTGGA AACGGTCGAGTGGAGTTACAACTGGGACTCCCTGTGTGGTGTGCATGTCCCTG	2986
human	GTTTCAGAAAC TGTCGACCAGAGT TAGAACGGAG---CCTCGGTGATGCGGACATCTCAG	2933
	*** **** * *	
mouse	GCTGGGGTTGTGTGACGACTCAGCACATC--TGCAGAGTGTTCTCTGTCCTGTGCCATGAC	3044
human	GGAAACTTGCCT---ACTCAGACAGCAGTGGAGAGTGTTCTGTTGAATCTGACAT	2989
	* * * * *	
mouse	ACTTCC-TGCTCAAGAAATTAGATGTGTCCTAACTGCGACGGGG-AGTAAAGACA--CTTCA	3100
human	ATTTGAATGTGAAGCATGTATACATTGATCAAGTTCGGGGGAATAAGACAGACCACA	3049
	* * * * *	
mouse	CCTCACACC---TGGAAGTGTTCTTAAAGGACGAGGGGAGTGCTACGTGGTCTGGGGAC	3156
human	CCTAAAACTGCCTTTCTGCTTCTCTTAAAGGAGAAGTAGCTGTGAACATTGCTGGGATAC	3109
	*** * * * *	
mouse	CTGATGTGTGGAATCC T---ATTGTTGTAA TAAAC TGACTAAAGGACTGGGGTAGCTC	3212
human	CAGATATTGAATCTTTCTTACTATTGGTAATAAACC TTGAT--GGCATTGGGCAACAG	3167
	* * * * *	
mouse	ATGTCCTCCATTTT-----	3224
human	TAGACTTATAGTAGGGTTGGGGTAGGCCATGTTATGTGACTATCTTTATGAGAATTTAA	3227
	* * * *	
mouse	-----	
human	AGTGGTTCTGGATATCTTTTAACTTGGAGTTTCATTTCTTTTCATGTGAATCAAAAAA	3287
mouse	-----	
human	AATTAAAGAGCCAAATACTTCTGAGACCTTGTTCATCTTTGCTGTATATCCCTC	3347
mouse	-----	
human	AAAATCCAGTTATTAACTTATGTGTTTCTTTTAAATTTTGTATGGATTCTTTAG	3407
mouse	-----	
human	ATTTAATGGTTCAAATGAGTCAACTTTGAGGGACGATCTTGAATATACCTACCTATTA	3467
mouse	-----	
human	TAAATCTTACTTTGTATTTGTATTT 3493	



**Supplementary figure 2 (lanes 1-2). Identification *ENPP1*-3'UTR-binding protein.**

REMSA was carried out using <sup>32</sup>P-labeled *ENPP1* probe in absence (lane 1) or presence (lane 2) of HEK293 cell extracts as described under “Methods.” The observed band shifting indicated the formation of RNA-protein complex (lane 2). **(lanes 3-12). HSP70/*ENPP1*-3'UTR binding specificity.** <sup>32</sup>P-Radiolabeled *ENPP1* probe (see “Methods”) was subjected to PAGE after incubating (lane 2-10) or not (lane 1) with HEK293 extracts. To examine protein/RNA binding specificity, increasing amount of HSP70 SPA-812 antibody (lanes 3–10) or total human IgG (lanes 11–12) were added to cell extracts/*ENPP1* probe mixture before running PAGE.

Makaa**V**g**i**d lgttyscvgv fch**g**K**V**E**I**I**A** **N**D**Q**G**R**T**T**P**S** **Y**V**A**F**T**D**I**E**R**L igdaak**N****V****A** **L**N**P****Q****N****T****V****F****D****A**  
**K**R**I**g**r****K****F****G****D** **P****V****V****Q****S****D****M****K****H****W** **P****F****Q****V****I****N****D****G****D****K** **P****K****V****Q****S****Y****K****G****E** **T****K****A****F****Y****P****E****E****I****S** **S****M****V****L****T****K****M****K****E****I** **A****E****A****M****L****G****P****V****T**  
**N****A****V****I****T****V****P****A****Y****F** **N****D****S****Q****R****q****a****t****k****D** **A****G****V****I****A****G****L****N****V****L** **R****I****T****N****E****P****T****A****A** **I****A****Y****G****L****D****R****t****g****k** gernvlifdl gggtfdsIl  
tiddgifevk **A****T****A****G****D****T****H****L****G****G** **E****D****F****D****N****R****L****V****N****H** **F****V****E****E****F****K****R****k****h****k** kdisqnkrav rrlrtacera krtlsstQa  
sleidslfeg idfytsitr**A** **R****F****E****E****L****C****S****D****L****F** **R****S****T****L****E****P****V****E****K****a** lrdak**L****D****K****A****Q** **I****H****D****L****V****L****V****G****G****S** Tripkvqk**L**  
**Q****D****F****F****N****G****R****d****l****n** **k****S****I****N****P****D****E****A****V****G** **Y****G****A****A****V****Q****A****R****I****L** **M****G****D****K****s****e****n****v****q****d** lllldvapls lgletaggvm talik**N****S****T****I**  
**P****T****K****Q****T****Q****I****F****T****T** **Y****S****D****N****Q****P****G****V****L****I** **Q****V****Y****E****G****E****R****a****m****t** **k****D****N****N****L****L****G****R****f****e** lsgippapgv pqievtfdid angilnvtat  
dkstgka**N****k****I** **T****I****T****N****D****K****G****L****S** **K****E****E****I****E****R****m****v****q****e** aek**Y****K****A****E****D****E****V** **Q****r****e****r****v****s****a****k****N****A** **L****E****S****Y****A****F****N****M****K****S** **A****V****E****D****E****G****L****K****g****k**  
iseadkkkv**l** **d****k****Q****E****V****I****S****M****L** **D****A****N****T****L****A****E****K****D****E** **F****E****H****K****k****e****L****E****Q** **V****O****N****P****I****I****S****G****L****Y** **Q****G****A****G****G****P****G****P****G****G** **F****G****A****Q****G****P****K****G****G****S**  
**G****S****G****P****T****I****E****E****V****D**

Supplementary figure 3. Amino acid sequence of HSP70. Uppercases show the peptides identified by tandem mass spectrometry.